# COMPARATIVE CYTOFLUOROMETRIC STUDY OF THE DNA CONTENT IN HIGH POLYPLOIDY CELL NUCLEI STAINED WITH ACRIDINE ORANGE AND FLUORESCENT SCHIFF-TYPE REAGENT



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# COMPARATIVE CYTOFLUOROMETRIC STUDY OF THE DNA CONTENT IN HIGH POLYPLOIDY CELL NUCLEI STAINED WITH ACRIDINE ORANGE AND FLUORESCENT SCHIFF-TYPE REAGENT

[Following is the translation of an article by A. A. Zotikov, Institute of Molecular Biology, USSR Academy of Sciences, Moscow, published in the Russian-language periodical Izvestiya Akademii Nauk SSSR, seriya Biol. (Bulletin of the Academy of Sciences, USSR, Biology Series), 1965, No 6, pages 921--925. It was submitted on 24 Nov 1964. Translation performed by Sp/7 Charles T. Ostertag Jr.]

With each year the use of fluorescence microscopy in biological investigations is spreading (DeLerma, 1958; Jaitinger, 1959; Meysel, Gutkina, 1961; Bertalanffy, 1962; Brumberg et al, 1963). In recent years increasing importance has been acquired by methods of quantitative cytofluorometric and histochemistry. Due to their exceedingly high sensitivity these methods make it possible to obtain useful information on intracellular processes and to detect low concentrations of biologically important substances in the cell (Ornstein et al., 1957; Chance, Thorell, 1959; Loeser et al., 1960; Brumberg et al., 1963; Barskiy, Ivanov, 1964).

Recently the fluorescent-histochemical method was proposed for exposing tissue polyaldehyde groups and, in particular, cell nuclei DNA, stained with the fluorescent Schiff-type reagent in the Feulgen reaction (Kasten, 1959). Fluorescence microscopic measurements of cell nuclei, carried out with this reagent, indicate the quantitative nature of determining DNA in situ (Bosshard, 1964). However, up until now this method has not been used to investigate cell nuclei with a high degree of ploidy.

In the present work we investigated high polyploidy nuclei of Protozoa, stained with the fluorescent Schiff-type reagent in the Feulgen reaction.

In this report a simultaneous examination is made of the problem of the feasibility of using a fluorochrome of acridine orange for the fluorescence microscopic determination of the content of DNA in cell nuclei. The use of identical cells made it possible to make a comparison of the results obtained.

As is known (Zelenin, 1961), acridine orange (AO) is used extensively in cytofluorometric and histochemistry for detecting nucleic acids.

It has been demonstrated that under specific conditions AO is selectively bound with nucleic acids and cell nucleoproteins (Meysel, Korchagin, 1952; Armstrong, 1956; Bertalanffy, Bickis, 1956; Schummelfeder et al., 1957).

Numerous observations showed that cell nuclei, stained with AO, fluoresced with a green or yellow-green color (with the exception of the nucleolus, which under specific conditions fluoresces a red color). Apparently the green or yellow-green fluorescence of the nuclei is caused by the binding of AO with double helical DNA (Borisova, Tumerman, 1964). In connection with this it was interesting to clear up to what degree, based on the intensity of fluorescence in the yellow-green area of the spectrum, it is possible to judge the content of DNA in cell nuclei stained with AO.

The objects of investigation were the ciliate protozoa Tetrahymena pyriformis, which possess a high polyploidy macronucleus (Elliot, 1959). For the experiments we used a culture of these protozoa, synchronized by the method of heat shocks (Scherbaum, Zeuthen, 1954). This method facilitates the study of cells in the various stages of their life cycle and makes it possible to obtain objects with a variable DNA content in the macronucleus (Ma). Measurements of the intensity of fluorescence of the cell nuclei were made with the help of a microscope-cytofluorometer which we assembled (Zetikov, 1964a). Fluorescence was excited by light with a wave length of 436 mμ, and measured in the range of 510--550 mμ.

For the investigation of cell nuclei stained with AO we used sufficiently low concentrations of dye ($3 \cdot 10^{-5}$ M), which is important for fluorometric measurements. However, under these conditions (pH 4.2--4.7, fixed time for staining no longer than 10 min.) both the DNA and the cell RNA fluoresced with practically the same green or yellow-green color. These observations indicated that the color of fluorescence of cells stained with AO is determined not only by the structure of the biopolymer, but also depends to a significant degree on the conditions of staining. Recently in a number of works, carried out in vitro, it was noted that complexes of AO both with DNA and with RNA in a number of cases possess practically identical spectral and fluorescent characteristics (Ranadive, Korgaonkar, 1960; Borisova et al., 1963). Since the nuclei of the culture used by us contain considerable amounts of RNA in addition to DNA, then for increasing the specificity of the reaction of AO with DNA the cells were treated with a solution of crystalline ribonuclease. The results of this are presented in figure 1. As is seen from this drawing, based on the degree of removal of RNA from the nuclei as a result of enzyme treatment, the intensity of their secondary fluorescence drops and constitutes approximately half of its initial value after a 45 minute hydrolysis in a solution of ribonuclease (RNAase) (0.5 mg/ml; 37°, pH6.5--7). If the time for hydrolysis is lengthened the intensity of fluorescence of the nuclei hardly changes. In connection with this, for making a judgment of

quantitative changes in the content of DNA in the nuclei of Tetrahymena pyriformis by the fluorescence microscopic method, we subsequently used cells from which the RNA was removed as a result of a 60 minute hydrolysis in a solution of RNAase. At specific times after the second synchronous division (after 20, 50 and 90 minutes) the cells were fixed with ethyl alcohol and stained with AO. The average time for a generation of cells was approximately 110 minutes. The results of the measurements are presented in figure 2. As is seen from this graph, the intensity of secondary fluorescence of the Ma increases throughout the life cycle of the cell, apparently, proportional to the amount of DNA in them. Together with this, these data show that it is hardly possible by this method to quantitatively determine the content of DNA in the nuclei of the cells being investigated.

The fact is that the DNA in the cell, in contrast to solutions, is found in a complex with protein. As we demonstrated earlier (Zotikov, 1964b), the basic protein, included in the DNA--protein make-up, exerts an influence on the intensity of fluorescence of cell nuclei stained with AO. This is apparently connected with the fact that the $NH_3^+$ -groups of AO react with the $PO_4^-$-groups of DNA, with which the protein is bound, and during the "liberation" of phosphate groups an increase in the binding of the dye takes place. As a result of the low optical density it is not possible to detect these small changes in the amount of dye by methods of absorption cytophotometry. However, changes in the DNA:protein ratio, which characterizes the DNA-proteid status to a certain degree, may be detected by the cytofluorometric method. In this manner the degree of intensity of fluorescence of cell nuclei, treated with ribonuclease and stained with AO, apparently reflects not only changes in the content of DNA, but also the condition of the desozyribonucleoprotein itself.

In another series of tests we carried out a fluorescence-microscopic quantitative determination of the DNA in the nuclei of the same type of cells, but treated with Schiff-type fluorescent reagent in the Feulgen reaction. In selecting this reagent we screened a number of fluorochromes; the most suitable for the quantitative analysis of nuclei turned out to be a reagent containing auramine O. In principle the method which we used for treating and staining the cells was similar to the one described earlier (Bosshard, 1964). After the cells were treated with this reagent (auramine O-$SO_2$) in the Feulgen reaction the nuclei fluoresced with a bright yellow-green color against a background of a dimly fluorescent cytoplasm. Measurings of the intensity of fluorescence of the nuclei were carried out in the same spectral range as in the work with AO. In the calculations the fluorescence of the cytoplasms was taken into consideration.

The results of the measurements are presented in figure 3,A. It can be seen from this drawing that throughout a large portion of the life

cycle of the cell the intensity of fluorescence of the Ma approximately doubled and apparently reflects the process of normal replication of DNA in the nuclei. A comparison of these data with the results of the measurements of cells stained with AO, presented in figure 2, points out the advantages of using the fluorescent Schiff-type reagent in the Feulgen reaction for determining the relative amount of DNA in cell nuclei. It should be noted that during the staining of cells with auramine O $SO_2$ in the Feulgen reaction, the protein which is included in the make-up of the DNA-proteid does not influence the degree of intensity of nuclear fluorescence.

The data obtained by using this method was also compared with the results of absorption cytophotometry of the nuclei of similar cells and at the same stages in their life cycle, but treated with fuchsine - sulfurous acid in the Feulgen reaction. We obtained a fully satisfactory agreement in the results of the tests carried out by both methods (figure 3, A and 3, B). However, the fluorescence microscopic method of determining the amount of substance in a cell still has significant advantages in comparison with the absorption method (Ornstein et al., 1957; Bosshard, 1964).

Quite conclusive proof in favor of the specificity of the fluorescent Schiff-type reagent for DNA were the tests which we carried out on the treatment of cells with DNAase, and also the tests on the blocking of the aldehyde groups with hydroxylamine after acid hydrolysis (Pira, 1962). In the first case there was a sharp reduction of intensity of fluorescence in the area of the nucleus, while in the second case the entire cell did not fluoresce.

Thus, these investigations showed that during staining of nuclei in the Feulgen reaction with fluorescent Schiff-type reagent, containing auramine O as the chromophore group, it is possible to determine the relative amounts of DNA even in high polyploidy nuclei, based on the intensity of fluorescence of the dye bound with the DNA. As regards acridine orange, it apparently can be used in quantitative cytofluorescent and histochemistry for investigating the condition of nucleoproteins, and in particular nuclear DNA-protein after the RNA has been removed from the cells.

## Conclusions

1. An investigation of the macronucleus of Tetrahymena pyriformis, stained with fluorescent Schiff-type reagent (auramine O-$SO_2$) in the Feulgen reaction, showed that the cytofluorometric method may be used to determine the relative amounts of DNA even in high polyploidy cellular nuclei.

2. When RNA has been removed from the cells it is possible to judge the condition of the DNA-protein based on measurements of the intensity of fluorescence of the cell nuclei, stained with acridine orange.

3. The two methods are complementary in the investigation of cell nuclei by the cytofluorometric method.

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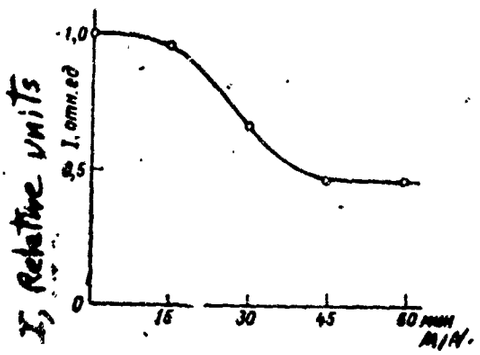


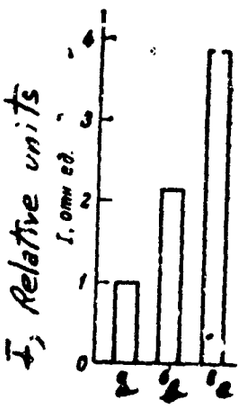


Fig. 1 Fig. 2

Figure 1. Changes in the intensity of fluorescence of cell nuclei, stained with AO, depending on the time of hydrolysis in a solution of ribonuclease.

Figure 2. Changes in the intensity of fluorescence of cell nuclei, stained with AO, at various stages in the life cycle of the cell (after the second synchronous division: a - after 20 minutes, b - 50 minutes, c - 90 minutes)

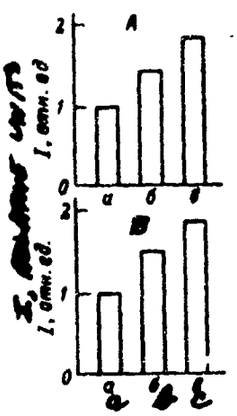


Figure 3. Changes in the relative content of DNA in cell nuclei, stained in the Feulgen reaction, after 10 minutes of hydrolysis in 1 N HCl at 60°. A - treatment of cells with auramine O-$SO_2$ ; along the axis of ordinates -- intensity of fluorescence of nuclei in relative units (I); B - the same with fuchsine-sulfurous acid, along the axis of ordinates -- relative average amount of DNA in the cell nuclei. Legend - same as figure 2.